



In vitro wound healing potency of methanolic leaf extract of *Aristolochia saccata* is possibly mediated by its stimulatory effect on collagen-1 expression



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ABSTRACT

Background: Identification and assessment of therapeutic potential of natural products derived from medicinal plants have led to the discovery of innovative and economical drugs to treat several diseases, including chronic wounds. *In vitro* cell based scratch assay is an appropriate and inexpensive method for initial understanding of wound healing potential of medicinal plant extracts. The current study was aimed at investigating the wound healing capacity of *Aristolochia saccata* leaf extract by using scratch assay as a primary model, where proliferative and migratory capabilities of test compounds could be monitored through microscopy studies. *A. saccata* is an evergreen climbing shrub belongs to the family Aristolochiaceae.

Methods: Methanolic extraction of the plant material was done using Soxhlet apparatus and the cytotoxicity of the extract on L929 cells was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. L929 is a human fibroblast cell line. *In vitro* scratch assay was performed to evaluate the wound healing properties of *A. saccata* leaf extract and possible mechanism of action was analyzed by flow cytometric expression studies of an extracellular matrix (ECM) factor, collagen type-1.

Results: MTT assay revealed that *A. saccata* leaf extract had no cytotoxic effect on the cells and at higher concentrations, the extract showed mild toxicity resulting in the death of just 2.88% cells. Scratch assay showed 34.05%, 70.00%, 93.52% wound closure at 12hrs, 24hrs and 48hrs of incubation respectively. These results were similar compared to positive control which showed 37.60, 56.41 and 99.05% of wound closure. Further, flow cytometry-based studies revealed that the *A. saccata* leaf extract induced the expression of ECM remodelling factor collagen-1.

Conclusion: Our study revealed the wound healing capabilities of *A. saccata* *In vitro*. Hence, *A. saccata* could be recommended as a potential source of wound healing agents.

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1. Introduction

Skin, the largest organ of human body, protects visceral organs from infection by microbes and injury. Wound healing mechanism is obligatory to regain the lost tissue and maintain tissue homeostasis. New tissue formation is a complex process, which involves multiple steps such as inflammation, angiogenesis, granulation tissue formation, re-epithelialization, and ECM reconstruction [1]. Upon injury in the skin, cells such as fibroblasts, keratinocytes, macrophages, and other immune cells rapidly proliferate and migrate towards the wound and initiate the complex healing process. Hence, migration of cells towards wound is one of the key phases of wound healing process and in general, it is governed by various stimulatory factors of tissue microenvironment [2].

Fibroblasts are most abundant cells in skin tissue and the major functions of these cells during wound healing include, rupturing of fibrin clots, generation of extracellular matrix (ECM) components and collagen structures that support the tissue homeostasis [3, 4]. Collagen synthesis and granulation tissue formation play critical role in wound contraction. For this reason, contemporary wound healing research is focused on the identification of new therapeutic agents, which has a stimulatory effect on the activation and modification of collagen producing fibroblasts [5].

Several *In vitro* and animal models are available to screen the wound healing nature of new therapeutic agents. Among them, fibroblast cell-based scratch assay is an inexpensive and well-established model, which supports the initial understanding of wound healing efficacy of new therapeutic agents [6].

Natural extracts have been playing fundamental role in the acceleration of wound healing process. However, the scientific evidence of their efficacy is limited. Hence, efforts to identify the bioactive compounds of medicinally important herbal extracts and their mechanism of action has always tremendous importance in the medical research [7]. *A. saccata* is an evergreen climbing shrub belonging to the family Aristolochiaceae. Over 500 species of this family have been identified [8] and the extracts of some of the species have been used as medicine for various diseases, though the scientific evidences are limited. Aristolochiaceae plants are known to treat arthritis, snake bites, wounds and skin diseases. They are also known to possess anti-cancer, anti-inflammatory [9], anti-feedant [10], muscle relaxant [11] properties. Although these medicinal properties of the plant are known traditionally, these properties have not been scientifically validated through *In vitro* or *In vivo* experiments. Specific medicinal properties of this plant, as evidenced by cellular and molecular biology experiments, are not reported. Therefore, we strived to validate the traditional claims and checked for the wound healing potency of the plant extract.

In the present study, the wound-healing efficacy of methanolic extract of *A. saccata* leaf was determined by *In vitro* scratch assay and its role in the stimulation of collagen-1 expression in L929 cells was studied by flow cytometry.

2. Results

2.1. Cytotoxicity effect of *A. saccata* leaf extract

Although plant extracts have been long studied for their medicinal properties, the cytotoxic effects of such extracts on the cell type of interest is sometimes neglected. However, in the recent times, there has been a growing trend in testing this critical component [12, 13]. Cytotoxic effect of *A. saccata* leaf extract on L929 cells was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were exposed to different concentrations of test compound for 48hrs and the cytotoxic effect of the extract was evaluated. The percentage viability of L929 cells at the highest treated concentration of *A. saccata* leaf extract was observed to be 97.12 ± 0.65 . The concentrations of *A. saccata* leaf extract used for treatment and their corresponding percentage cell viability were tabulated in Table 1 and represented in Fig. 1 and 2. These results indicated that the extract was not cytotoxic and

Table- 1

Cell viability effects of *A. saccata* extract in L929 cell line.

Culture conditions	% of cell viability
Vehicle Control	99.33 ± 0.42
31.25	99.49 ± 0.10
62.5	99.06 ± 0.34
125	97.70 ± 0.31
250	97.44 ± 0.14
500	97.12 ± 0.65

could be assessed for their medicinal properties.

2.2. Fibroblast cell migration was induced by *A. saccata* leaf extract

Activation, proliferation and migration of fibroblasts are the primary steps in wound healing, where multiple cell types and other micro environmental factors are involved. Scratch assay is a widely applied *In vitro* technique for understanding the wound healing capabilities of medicinally important compounds [14]. In the current study, L929 cells were treated with 125 $\mu\text{g/mL}$ of *A. saccata* extract for 48hrs. Cell migration at 0, 12, 24, 48 h were captured and wound closure distance was calculated by Image J software. The results indicated that *A. saccata* leaf extract, at 125 $\mu\text{g/mL}$, closed the gap created by the scratch by 93.525% in 48 h. Percentage wound closure at different time intervals in untreated, extract treated and control drug-treated cells have been represented in Fig. 3. *A. saccata* leaf extract induced the migration of L929 cells resulting in wound closure. In the standard-drug treated cells, 99.05% of the gap was closed at 48 h. Fig. 4 shows the microscopic images of untreated, standard drug-treated and extract-treated L929 cells. The photographs show increased cell migration in the control drug-treated cells and extract treated cells

2.3. Collagen type 1 expression was increased dose dependently

Collagen-1 is the major protein of the extracellular matrix (ECM) and is not only involved in the formation of ECM during the wound healing process, but also enhances cellular proliferation, migration, differentiation and synthesis of other essential proteins from the surrounding cells [15, 16]. In the current study, the expression of Collagen type 1 was analysed by flow cytometry after 48 h of treatment of L929 cells with 125 $\mu\text{g/mL}$ of *A. saccata* leaf extract or 10 ng/mL of human Epidermal Growth Factor (hEGF). Experimental results showed increased expression of collagen type 1 in *A. saccata* leaf extract treated and hEGF treated cells. The percentage of cells that expressed collagen type 1 in the extract treated cells was 69.92 % and in hEGF treated cells was 90.69 % of cells. The flow cytometric analysis of Collagen type 1 expression and the quantification of the expressing cells in untreated, extract treated and hEGF treated cells have been represented in Fig. 5 and 6, respectively. These results indicate a clear increase in expression of Collagen type 1 in extract-treated cells compared to the untreated control indicating that the extract enhances Collagen type 1 expression in L929 cells, possibly thereby enabling the wound healing process.

3. Discussion

Wound healing is a complex mechanism and a variety of plants used traditionally in folk medicine have been ethnopharmacologically validated for their wound healing properties. A number of *In vitro* studies have been conducted with the crude plant extracts or isolated secondary metabolites to understand their extended use in wound healing [17]. Several medicinal properties like arthritis, snake bites, wound healing and skin diseases, anticancer, anti-inflammatory [9], antifeedant [10], muscle relaxant [11] properties of plants belonging to the Aristolochiaceae family have been reported both in traditional and modern medical research deeming the plants to be medicinally relevant and important.

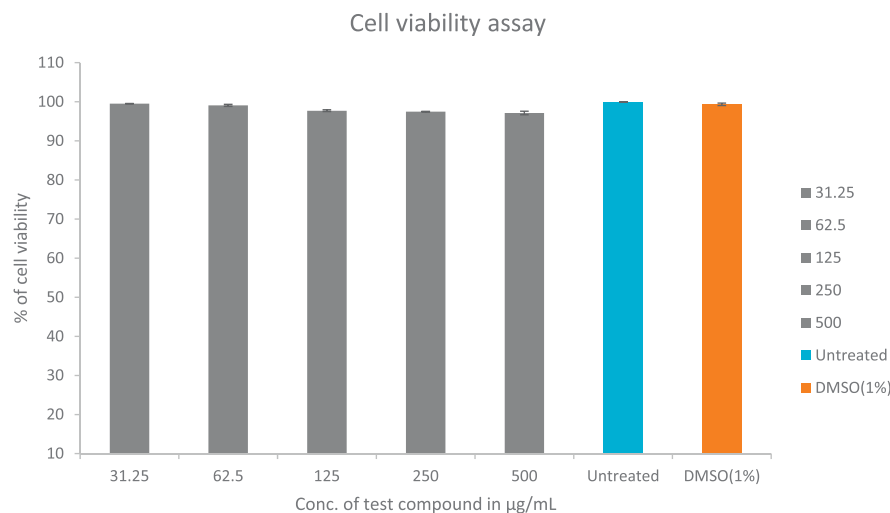


Fig. 1. The effect of *A. saccata* on L929 cell line viability was determined by MTT assay method. Each bar graph represents % viability of L929 cells against 31.25 to 500 µg/mL concentrations of *A. saccata* extract after 48 hrs exposure. Untreated cells were negative control and 1% Dimethyl Sulfoxide (DMSO) as vehicle control. Experiments were performed in triplicates and data was shown as mean $\pm$ SD.

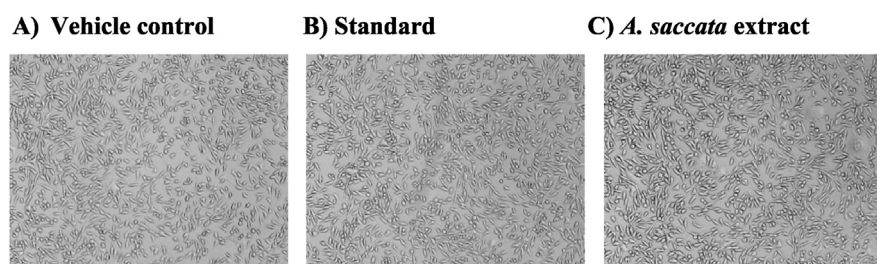


Fig. 2. Images of L929 cell line in inverted light microscopy after the exposure to *A. saccata* extract. From 'A' to 'C' where A) vehicle control (1% DMSO) B) 5 µg of standard drug Cipladine and C) 500 µg/mL concentration of *A. saccata* extract. After incubation of 48 hours *A. saccata* extract displayed no toxicity.

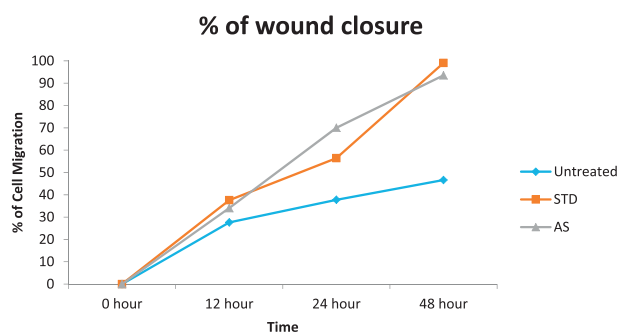


Fig. 3. Percentage of cells migrated towards the wound and involved in wound closure. Migration of cells in the absence or presence of *A. saccata* leaf extract. Blue: cells with culture medium alone; Orange: 5 µg/mL of standard drug Cipladine; Grey: 125 µg/mL of *A. saccata* leaf extract.

Although these medicinal properties of the plant are known traditionally, these properties have not been scientifically validated. Therefore, in this study, we evaluated the wound healing properties of *A. saccata* by *In vitro* assays and expression studies. A typical wound healing process encompasses complex cellular changes that include inflammation, angiogenesis, re-epithelialization, granulation tissue formation, and remodeling of extracellular matrix [2]. In the initial stages of wound healing, fibroblasts play a vital role by actively proliferating, migrating to wound area and inducing the synthesis of new extracellular matrix (ECM), and thick actin myofibroblasts [18]. Fibroblast cell lines like TIG119 [19], NHDF [20],

HDF-D [20], L929 [21, 22] etc. have been employed to assess the wound healing potencies of several compounds *In vitro*. Besides plant extracts, herbal formulations made of a combination of plant extracts have also been tested using these cell lines, particularly the L929 cells [21]. In other words, the migratory and proliferative abilities of the fibroblasts are pivotal to wound healing. In order to assess the wound healing potency of the methanolic extract of *A. saccata* leaves, we performed scratch assay, a widely used *In vitro* assay in wound healing studies [14], on the fibroblast cell line L929. In our study, we observed that L929 cells migrated better toward the artificially created wound when treated with the *A. saccata* leaf extract. This suggests that the extract accelerates wound healing by inducing the migration of fibroblasts. A similar study on another species of the same family, *A. bracteolate*, reported that its methanolic extract stimulated the migration of fibroblasts and keratinocytes and enhanced the expression of wound healing related genes [23].

Further, fibroblasts synthesize ECM components and growth factors like Collagen, FGF, EGF, TGF- β etc., which form the key regulators of wound healing process and are involved in the regeneration of injured ECM [4]. Of these, collagen is a key component of the ECM and its synthesis is proportional to the amount of hydroxyproline synthesized [4, 18, 24–27]. Collagen type 1 is a crucial factor and is imperative to induce major cellular events like extracellular matrix remodeling during the wound healing process [24, 25, 26, 27] and angiogenesis [28]. Moreover, the strength and integrity of the newly formed blood vessels is directly linked to the amount of collagen synthesized by the fibroblasts [18]. Studies focused on plants for wound healing properties have also

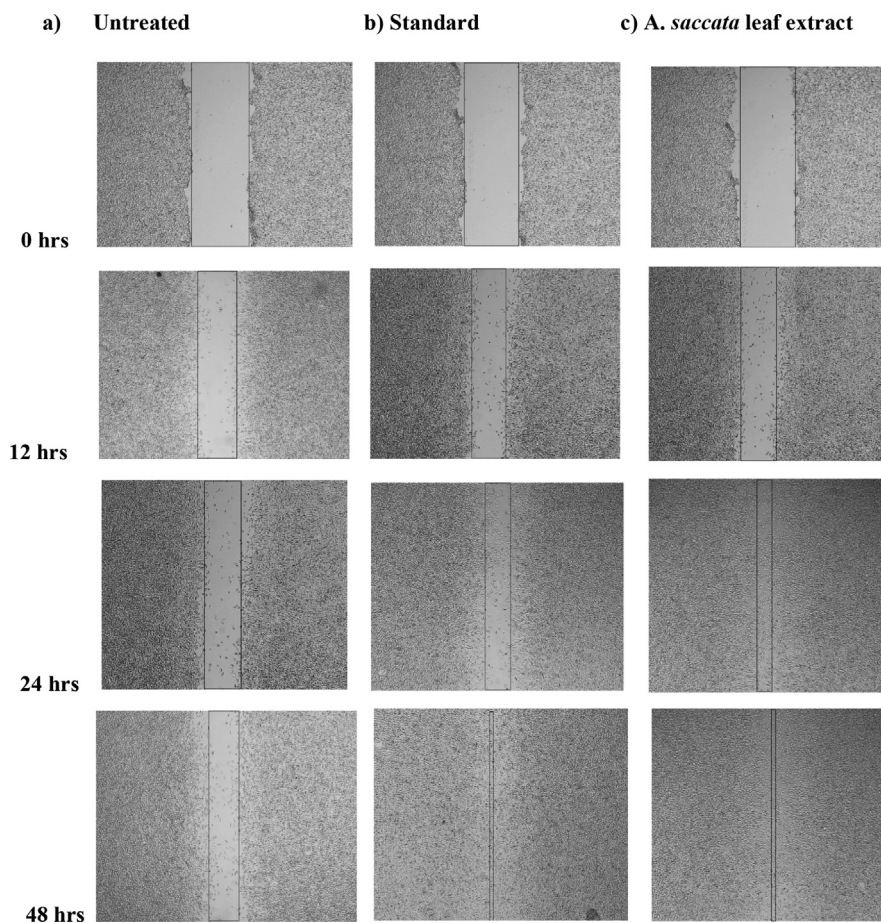


Fig. 4. Microscopical images representing the *In vitro* wound healing nature of methanolic extract of *A. saccata* leaf: L929 cells were incubated in presence or absence of *A. saccata* leaf extract and standard drug Cipladine and images were captured at 0, 12, 24 and 48 hrs. (a) Negative control, (b) 5 μ g of positive control Cipladine (c) 125 μ g/ml of *A. saccata* leaf extract. The boundaries of the scratched wounds were determined by the dark lines.

demonstrated their ability to increase collagen production. For example, the oral and topical administrations of *Centella asiatica* extract not only showed increased collagen synthesis but also better maturation and crosslinking of collagen in rat models [29]. Also, Shirwaikar *et al.* reported that the use of *A. bracteolata* increased the rate of wound contraction and there was a significant increase in the hydroxyproline content, which is an indication of collagen levels [30]. Adams *et al.* reported a few native plants of Australia that differentially induce Collagen I and Collagen III *In vitro* upholding them as a useful source of wound healing compounds. In this report, the bioactive compounds that bring about the induction of collagen were also elucidated, suggesting that plants induce Collagen expression through their bioactive compounds [31]. Therefore, we studied the expression of Collagen type 1 in the untreated, *A. saccata* extract treated and control molecule-treated L929 cells. In our study, the expression of Collagen type 1 was found to be upregulated on treating L929 cells with the *A. saccata* extract, suggesting that *A. saccata* possibly enhances the expression of Collagen type 1, thereby initiating the migration of fibroblasts bringing about wound healing. This indicates that *A. saccata* has potential wound healing properties and can be used to extract lead molecules in the discovery of wound healing agents. The phytochemical analysis of other well-known wound healing plants reveal the possible role of these phytoconstituents especially flavonoids and triterpenoids in wound healing. These phytochemicals have been documented to possess astringent, free radical

scavenging and antioxidant properties, which are known to aid wound healing process [32, 33]. Another possible mechanism is that the plant extracts increase the proliferation of fibroblasts cells and in turn increase the production of collagen in the affected area. This was observed through increase in DNA, total protein and total collagen content of granulation tissues in wounded rat models treated with plant extracts [34].

The extract was also tested for its cytotoxicity using MTT assay. This cytotoxicity assay is based on the idea that early screening of any biological material for toxicity may help in the evaluating its biological and therapeutic relevance. Assessing the cytotoxic effects of the plant extract on the cells or an *in vivo* model is critical as some plant metabolites might have toxic effects on the cells because of their intermolecular interactions in the cells. This is indicated by a measure of the half maximal inhibitory concentration (IC₅₀) value. A high IC₅₀ value is representative of the fact that high concentration of the extract is essential to cause detrimental effects on the cell, whereas a low IC₅₀ value is indicative of the cytotoxic ability of the extract at smaller doses [35]. Previous literature suggests that an IC₅₀ value of 100 μ g/mL may be possibly toxic to cells [36]. Further, American National Cancer Institute (NCI) has set the IC₅₀ limit of 30 μ g/mL concentration for the extract to be considered toxic to the cells [37]. In our study, L929 cells were treated with extract concentrations much higher than these recommended thresholds and the percentage

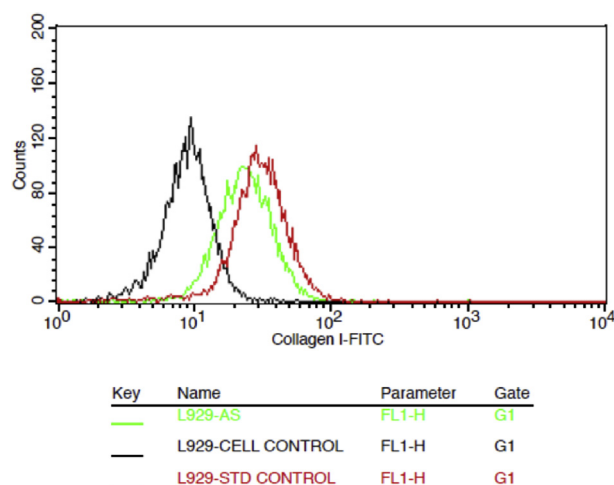


Fig. 5. Flow cytometry based expression studies of collagen-1. L929 cells were exposed to 125 $\mu\text{g/ml}$ and 10 ng/ml of *A. saccata* leaf extract and hEGF respectively for 48hrs. a) black-untreated cells; b) green – *A. saccata* leaf extract; c) red-hEGF.

viability of the cells was not affected much. This supports our claim that *A. saccata* extracts can be used in for wound healing without the fear of toxicity. Nevertheless, the scope of these claims is limited to the highest concentration of extract and the cell line type used in the study. A future study with an *in vivo* model, a different cell type and/or with higher concentrations may prove to be different. However, our study is a step forward in adding ethnopharmacological validation to the use of *A. saccata* in wound healing cases.

4. Materials & methods

4.1. Chemicals and reagents

Dulbecco's Modified Eagle's medium (DMEM) (#AL219A, Himedia), Fetal Bovine Serum (#RM10432, Himedia), Dulbecco's Phosphate Buffered Saline (DPBS) (#TL1006, Himedia), Mouse Anti-Human collagen I-Fluorescein isothiocyanate (FITC) antibody (#FCMAB412F, Merck),

Cipladine (Cipla Ltd), hEGF (#11376454001, Roche) MTT Reagent (#4060 Himedia), DMSO (#PHR1309, Sigma), Fluorescent Activated Cell Sorter (FACS) Calibur (BD Biosciences, INDIA), Microplate reader (#EC800, Biotek).

4.2. Methanolic extraction of leaves

Fresh leaves of *A. saccata* were shade dried, finely powdered using a mortar and pestle, and 100 g of the powder was used for methanolic extraction. The powder was mixed in 200 mL of methanol and was continuously agitated for 24 h using a magnetic stirrer. The extract was then filtered through Whatman no 1 filter paper and filtrate was concentrated in rotary evaporator at 40 °C. The extract was stored in dry tubes until further use. A stock solution of the extract was prepared in DMSO and working concentrations were prepared by diluting the stock in DMEM with 10% Fetal Bovine Serum.

4.3. Cell culture

The Mouse fibroblast cell line L929 was procured from NCCS, Pune and cultured in DMEM, premixed with 10% Fetal Bovine Serum and antibiotics (streptomycin 100 U/mL and penicillin 100 U/mL). The cells were maintained at 37 °C with 5% CO₂ in a humidified incubator and were passaged when they reached 80% confluency. Cells were counted using a Hemocytometer. Viability was calculated to seed the cells at appropriate densities, to perform the assays.

4.4. Cytotoxicity studies

The cytotoxicity of *A. saccata* leaf extract on L929 cells was evaluated by MTT assay [30]. Briefly, L929 cells were seeded in a 96-well plate at an initial seeding density of 2×10^4 cells/well/200 μL of DMEM and were cultured for 12 h. The cells were then treated with different concentrations of *A. saccata* leaf extract (31.25, 62.5, 125, 250, and 500 $\mu\text{g/ml}$), and were incubated for 48 h at 37 °C and 5% CO₂. Post incubation, the spent medium was removed and 20 μL of 5 mg/mL of MTT reagent was added to the cells and incubated for 2 h in the CO₂ incubator. The formazan crystals were solubilized with 100 μL of DMSO and absorbance at 570nm was determined using a microplate reader. The cells treated with

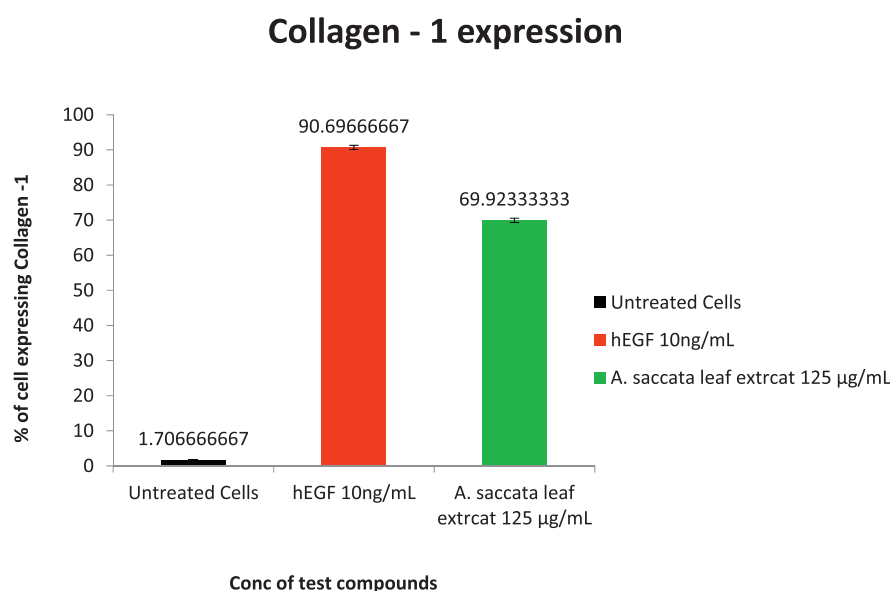


Fig. 6. Bar graphs represent the % of L929 cells expressing collagen-1 upon treatment with 125 $\mu\text{g/ml}$ of *A. saccata* leaf extract and 10 ng/ml of hEGF for 48 hrs a) black-untreated cells; b) green – *A. saccata* leaf extract; c) red-hEGF. The studies were conducted in triplicates and 10000 events were measured for each flow cytometry run.

DMEM alone were considered as negative control and 100% viable. The percentage of cell viability was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean absorbance of test sample}}{\text{Mean absorbance of negative control}} \times 100$$

Percentage of cell viability was plotted against concentrations of test samples. Three sets of experiments were performed in triplicate in and the data were presented as mean $\pm$ SD (n = 3).

4.5. Scratch assay

The wound healing capabilities of the *A. saccata* leaf extract was assayed by performing *In vitro* cell migration studies on L929 cells by a previously described method [14]. Briefly, 2×10^5 cells/mL were seeded in 6-well plates and were cultured overnight. Cells were then washed with Delbuccho's Phosphate Buffered Saline (DPBS) and a scratch was made with a sterile 200 μ L tip. The detached cells and other cellular debris were removed by washing the cells with DPBS. The cells were treated with 125 μ g/mL of *A. saccata* leaf extract and 5 μ g/mL of positive control, Cipladine [17] and incubated for 24 h. Cipladine is a standard drug that is used in wound healing [17, 32]. Untreated cells were negative control. The cell migration and morphological changes of cells were observed in the images taken by inverted microscope, equipped with digital camera. The experiments were performed in triplicate (n = 3). The width of the scratch and wound closure at different time intervals (0, 12, 24 and 48hrs) was analyzed by Image J software.

4.6. Flow cytometry

L929 cells were seeded in 6 well plate at an initial density of 2×10^5 cells/ml and were treated with 125 μ g/ml of *A. saccata* leaf extract or 10 ng/mL of hEGF. After 48 h of incubation, cells were trypsinized and washed with DPBS. Cells were fixed and permeabilized with 70% ice cold methanol at -20 °C and stained with Mouse Anti-Mouse Collagen I-FITC antibody by incubating at room temperature in dark for 30 min. FACS Calibur was used to evaluate the expression of collagen-1 and data was analysed by CellQuest Pro software.

All studies were conducted in triplicates and results expressed as mean $\pm$ SD (n = 3).

4.7. Statistical analysis

All the experiments were conducted in triplicates, and the results are expressed as mean percentage inhibition $\pm$ standard deviation (SD) (n = 3). Statistical significance was determined by one-way analysis of variance, followed by Bonferroni post hoc test for multiple comparisons, and $p < 0.05$ was considered statistically significant. All statistical analyses and IC₅₀ values determination were carried out in GraphPad Prism (version 3.1) software, GraphPad Software, 2365 Northside Dr. Suite 560, San Diego, CA 92108.

5. Conclusion

In conclusion, the methanolic extract of *A. saccata* leaf enhanced wound closure in L929 cells and expressed higher levels of Collagen type 1. Further, the extract was found to have no cytotoxic effect. These data suggest that *A. saccata* has possible wound healing properties and can be a plausible source for the extraction of natural wound healing compounds.

Declarations

Author contribution statement

Srinivasa Rao Bolla: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Janardhana Papayya Balakrishna, Shiva Shankar Reddy Gollapalli: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Abeer Mohammed Al-Subaiei, Reem Yousuf Al-Jindan, Vishnu Priya Veeraraghavan, Padma Kanchi Ravi, Joel Palpath Joseph, Aruthra Arumugam Pillai: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Surapaneni Krishna Mohan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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